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Use of a Signature Nucleotide Sequence of Hepatitis C Virus for Detection of Viral RNA in Human Serum and Plasma

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The nucleic acid sequence of the putative 5′-untranslated (5PUT) region of hepatitis C virus (HCV), determined for samples obtained from a variety of geographic origins, was found to be over 98% conserved among all isolates. On the basis of this signature sequence for HCV, a viral RNA assay was developed by using cDNA synthesis with reverse transcriptase, followed by polymerase chain reaction (PCR). The new assay was compared with the Ortho-Chiron C100-3 HCV enzyme-linked immunosorbent assay to research radioimmunoassays for antibodies to the C33c and C22 HCV antigens and to the first reported set of HCV PCR primers designed from the NS3 domain. Plasma samples from 16 Japanese patients with non-A, non-B hepatitis (NANBH) and 16 immunoassay-positive blood donors from the United States were investigated. The 5PUT PCR primers were found to be superior to the NS3 primers in sensitivity and specificity (15 of 25 versus 3 of 25 of the C100 enzyme-linked immunosorbent assay-positive samples, respectively). Samples from two C100-negative patients with acute NANBH were found to react with the 5PUT primers but not with the NS3 primers. Also, two of three patients with chronic NANBH converted from reverse transcriptase PCR positive to negative after interferon treatment. Although the clinical significance of the presence or absence of HCV RNA in samples from patients is not fully understood, the use of probes and primers from the 5PUT region (as opposed to primers from other segments) should not lead to false-negative results due to nucleic acid sequence variations in viral isolates.

Hepatitis C virus (HCV) is the major causative agent of posttransfusion and sporadic non-A, non-B hepatitis (NANBH) in the world (1, 6, 23, 25). In the United States, a 1 to 12% incidence of the disease has been observed among transfusion recipients (8); however, transfusion-mediated transmission may account for only 5 to 10% of NANBH (2).

Recently, HCV has been molecularly cloned and fully sequenced (4, 5, 13, 16). A cDNA clone was used to identify and characterize the positive-strand RNA genome. Nucleotide sequencing of multiple overlapping clones revealed one large open reading frame which could encode 3,011 amino acids within the approximately 10-kb genome. A segment designated C100 from a putative nonstructural domain within the open reading frame has been molecularly expressed as a fusion protein in *Saccharomyces cerevisiae* and used to develop an immunoassay (23). The anti-C100 assay has been used to show that although the frequency of anti-HCV antibodies in donor populations throughout the world is low (0.3 to 1.4%), some 80% of patients with chronic NANBH worldwide harbor antibodies to C100 (37).

The relationship between the presence of anti-C100 antibodies and viremia is unclear. Because there is a long delay in seroconversion (mean, 15 weeks [1]), viremic individuals can be C100 enzyme-linked immunosorbent assay (ELISA) negative. As a result, considerable effort is being expended to identify additional antigenic markers and develop methods to detect HCV RNA. Recently, two other portions of the major HCV open reading frame, C33c (derived from NS3) and C22 (from the core), have been expressed and used to develop immunoassays (22).

For detection of HCV RNA, we have sought to identify a nucleotide sequence in the viral genome that is well conserved among viral isolates throughout the world (13), i.e., a signature HCV nucleotide sequence. A further extensive analysis of the nucleotide sequence from the putative 5′-untranslated (5PUT) region of HCV from a geographically diverse collection of isolates is reported here. Comparison of the sequences obtained suggests that probes and primers designed from this segment of HCV should be extremely specific. Results of a cDNA synthesis-polymerase chain reaction (PCR) (30) (reverse transcriptase [RT]-PCR) assay for HCV RNA based on the 5PUT sequence are presented and compared with those of a similar method using primers based on the NS3 region of HCV (40).

MATERIALS AND METHODS

RNA isolation and cDNA synthesis. For RT-PCR analysis, 100 μl of serum or plasma was treated with 300 μl of digestion solution containing 1.33 mg of proteinase K (Boehringer) per ml, 0.67% (wt/vol) sodium dodecyl sulfate (SDS), 13.3 μg of MS2 RNA (Boehringer) per ml, 0.167 mM NaCl, 1.67 mM EDTA, and 6.7 mM Tris hydrochloride (pH 7.5). After incubation at 55°C for 1 h, the solution was extracted with equilibrated phenol, phenol-chloroform-isoamyl alcohol (vol/vol/vol; 25:24:1), and chloroform-isoamyl alcohol (vol/vol; 24:1). The sample was then split into two portions and ethanol precipitated. Double-stranded cDNA synthesis was then conducted with primer 5PUTc1-a (Table 1) essentially as described for the Bethesda Research Laboratories cDNA kit (catalog no. 8267SA), by using Moloney murine leukemia virus RT. Both first- and second-

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TABLE 1. Oligonucleotide probes and primers used

Sequence name-sense[a]	Sequence (5′–3′)	Use	Nucleotide positions[b]
5PUTc1-a	CCCAACACTACTCGGCTAG	cDNA synthesis	−74/−92
5PUT1-a	TTGCGGGGGCACGCCCAA	Antisense PCR primer	−98/−115
5PUT2-s	CCATGAATCACTCCCCTGTGAGGAACTA	Sense PCR primer	−285/−312
5PUTp1-s	GCCATGGCGTTAGTATGAGTGTC	Probe for Southern analysis	−238/−260

[a] S, sense; a, antisense.
[b] With reference to prototype HCV-1 (Fig. 1).

strand syntheses with RNase H and polymerase I were conducted prior to PCR.

PCR. Appropriate handling and isolation conditions were adhered to during all processing steps (24). The PCR was carried out under mineral oil in 100 μl of a solution containing 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus), 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.002% (wt/vol) gelatin, each deoxynucleoside triphosphate at 200 μM, and primers 5PUT1-a and 5PUT2-s (5PUTs/a primers [Table 1]; a similar set of primers is reported in reference 13) at 0.5 μM. After an initial denaturation step at 94°C for 5 min, five cycles of 94°C for 2 min, 50°C for 2 min, and 72°C for 3 min was followed by 30 cycles at 94°C for 1.5 min, 60°C for 2 min, and 72°C for 3 min and, finally, 7 min of elongation at 72°C (Thermal Cycler; EriComp, San Diego, Calif.). All RT-PCR reactions were performed in duplicate. Each run contained a negative standard water sample and a known HCV-negative human serum control that were subjected to the RNA extraction procedure. A control HCV RNA-positive serum sample was also employed. PCR was also done with the 36 and 37b HCV primers as described by Weiner et al. (40).

Detection of amplified product. One-tenth of the PCR reaction was subjected to 8% polyacrylamide gel electrophoresis. The gel was then stained with ethidium bromide and photographed at 302 nm. Subsequently, the material was transferred to a nylon membrane in 10× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate). The blot was then hybridized with a 5′-end ^{32}P-labelled probe (5PUTp1-s) for 12 h at 42°C in 0.5 M sodium phosphate (pH 7.2)–1 mM EDTA–7% SDS–1% bovine serum albumin. After being washed at 55°C with 2× SSC–0.1% SDS for 15 min, the blot was autoradiographed with intensifying screens at −70°C overnight.

DNA sequencing. For sequence analysis of PCR products, the reactions were subjected to gel electrophoresis as described above, stained, cut out, eluted, and purified with Prep-A-Gene (Bio-Rad no. 732-6010). The double-stranded DNA was melted at 95°C for 3 min and then quickly chilled on ice. Subsequently, the products were subjected to dideoxy-chain termination sequencing by using *Bst* polymerase (Bio-Rad) to catalyze the polymerization at 65°C and [α-^{32}P]dATP.

Nucleotide sequence accession numbers. The nucleotide sequences described here have been assigned GenBank accession no. M74243 to M74255.

RESULTS

HCV nucleotide sequence comparison. By using RT-PCR to generate sufficient quantities of viral nucleic acid, nucleotide sequences in the 5PUT region of HCV were determined for isolates from the United States (FF25897, FF25905, and FF25910), Japan (J1, J14, 184, 217, NB021, and NB011), and Italy (I1, I24, I124, and I128) (Fig. 1). A minimum sequence homology of 98% was observed among all of the isolates. No insertions or deletions were found compared with the prototype HCV-1 sequence (16).

It is unlikely that mutations in a PCR product would be observed by direct sequencing (21); however, to check the validity of the sequences obtained, one sequence (J1) was determined twice from two separate RT-PCR amplification. The nucleotide sequences were identical and contained the same differences from the prototype HCV-1. These nucleotide variations likely reflect the presence of different HCV strains in a single carrier population and not *Taq* polymerase-generated errors incorporated during PCR. It is common for RNA viruses to exist as heterogeneous populations of nonunique (but related) genomes (31). When M13 sequencing of cloned PCR products was employed, differences in the sequences of clones of the same isolate that were probably due to both *Taq* polymerase and population heterogeneity (data not shown) were often noted. As a result, we preferred the direct sequencing methods to obtain an average nucleic acid sequence. In our studies, double-stranded PCR product sequencing has proven more reliable than asymmetric methods (17).

HCV RNA detection in Japanese clinical specimens. For RT-PCR detection of HCV, a set of primers was synthesized from the 5PUT region (5PUTs/a). These primers were compared with the 36-37b primer set employed by Weiner et al. (40). Immunoassays for antibodies to the C100, C33c, and C22 antigens were also employed. A panel of 16 Japanese plasma samples from patients with NANBH were tested (Table 2). Virtually all samples from patients with chronic hepatitis and liver cirrhosis were clearly positive for antibodies against all three antigen markers. Whereas all but four of this group were positive with the 5PUTs/a PCR primers, only four samples were positive with the 36-37b primers. Each of the 36-37b-positive samples was also positive with the 5PUTs/a primers. Two chronic patients were negative with both sets of PCR primers. Consistent with a long delay in seroconversion (1), only one of the two samples from Japanese patients with acute NANBH (348 and 349) was positive with only one of the immunoassays; however, both samples were positive with the 5PUTs/a PCR primers.

The lack of an RT-PCR signal for samples 317 and 335 is particularly noteworthy. Both are follow-up samples from patients (identified as patients 1 and 2, respectively) who had received alpha interferon treatment in a Japanese clinical trial. A positive 5PUTs/a RT-PCR signal was found for samples taken at the initiation of treatment (Table 2). In contrast, patient 3 was 5PUTs/a RT-PCR positive before and after interferon treatment. None of the immunological markers were lost in the three patients (or diminished; data not shown) immediately after interferon treatment. The relationship between the RT-PCR and immunoassay results as a

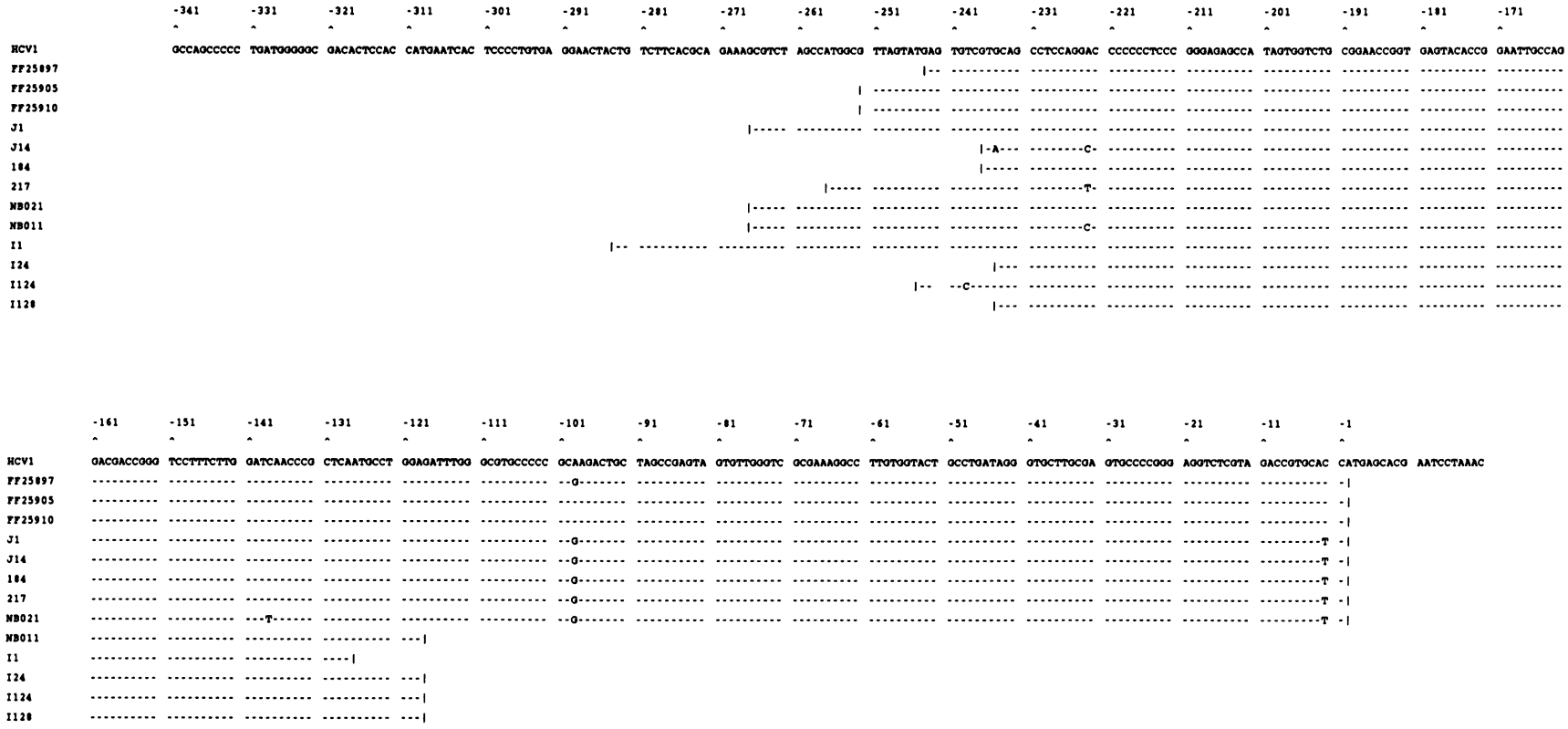

FIG. 1. Comparison of the 5PUT nucleotide sequences of HCV isolates from the United States, Japan, and Italy. Dashes indicate bases unchanged from the prototype HCV-1 sequence. The bars at the beginnings and ends of the dashes indicate the limits of the sequence information obtained.

TABLE 2. Results of ELISA, radioimmunoassay, and PCR analyses of Japanese samples

Sample	C100 ELISA	Radioimmunoassay with:		PCR with:		Comment(s)[a]
		C33c	C22	5PUTs/a	36-37b	
NB017	+	+	+	−	−	CH
NB005	+	+	+	−	−	CH
184	−	+	+	+	+	CH
348	−	−	+	+	−	AH
349	−	−	−	+	+	AH
214	+	+	+	+	+	LC
NB003	+	+	+	+	+	LC
217	+	+	+	+	−	AH
NB021	+	+	+	+	−	CH
NB011	+	+	+	+	−	LC
312	+	+	+	+	−	CH, IFN start, patient 3
313	+	+	+	+	−	CH, EFN end, patient 3
316	+	+	+	+	−	CH, IFN start, patient 1
317	+	+	+	−	−	CH, IFN end, patient 1
334	+	+	+	+	−	CH, IFN start, patient 2
335	+	+	+	−	−	CH, IFN end, patient 2

[a] CH, chronic hepatitis; AH, acute hepatitis; LC, liver cirrhosis; IFN, alpha interferon treatment.

function of long-term clinical status requires further investigation.

HCV RNA detection in U.S. blood donors. A set of plasma samples from the United States was also studied (Table 3). As opposed to the Japanese panel, the U.S. material was collected by screening apparently healthy blood donors. Although all but one of the samples were positive for both the C33c and C22 markers, four samples were negative for the C100 antigen. Only 8 of 16 were positive with the 5PUTs/a PCR primers and 1 of 16 was positive with the 36 and 37b primers. As for the Japanese samples, all 36-37b-positive samples were also positive for the 5PUTs/a primers.

TABLE 3. Results of ELISA, radioimmunoassay, and PCR analyses of U.S. samples

Sample	ELISA C100	Radioimmunoassay with:		PCR with:	
		C33c	C22	5PUTs/a	36-37b
84-017781	+	+	+	+	−
FF25897	+	+	+	+	−
FF25905	+	+	+	+	+
FF25910	+	+	+	+	−
FF25912	−	+	+	+	−
FF25926	−	+	+	+	−
FF25934	−	+	+	−	−
FF25946	+	+	+	−	−
LL57366	+	+	+	−	−
LL57382	+	+	+	−	−
LL57403	+	+	−	−	−
84-016953	+	+	+	−	−
84-017786	+	+	+	+	−
96696	+	+	+	−	−
96727	+	+	+	+	−
LL57385	−	+	+	−	−

Comparison of RT-PCR methods. A comparison was made between detection of PCR products by ethidium bromide staining and detection by Southern analysis. After cDNA was synthesized from representative samples of the Japanese and U.S. panels, the material was amplified by PCR with the 5PUTs/a and 36-37b primers, run on polyacrylamide gels, stained with ethidium bromide, or blotted and hybridized with a ^{32}P-labelled probe (Fig. 2). In Fig. 2A, it can be seen that although the anticipated band was observed for all 5PUTs/a-positive samples, occasionally several other products were obtained. For samples FF25897 and 316, larger and smaller bands, respectively, were found that reacted specifically with the probe in the Southern blot (Fig. 2B). We have occasionally noted multiple primer fragments at the termini of PCR products during sequencing studies, and this probably accounts for the larger bands. Associated with 5PUTs/a RT-PCR-negative sample FF25946 was an ethidium bromide-stainable band near the size expected for the amplified product that did not react with the probe on the blot. With the 5PUTs/a primers, samples have rarely been detected with a Southern blot and not with staining. In contrast, we often observed 36-37b RT-PCR positivity only with blotting, and then only with a nick-translated probe (Fig. 2C and D). Also, many more nonspecific RT-PCR products were observed when ethidium bromide staining was used.

DISCUSSION

The remarkable homology of the 5PUT region of the HCV genome is suggestive of a necessary role in viral replication (13). This high level of conservation is in contrast to limited sequence comparisons that have been made within portions of the putative nonstructural regions of the virus (9, 20, 32, 33, 39).

Few patterns in the sequence variations were found; however, all five of the Japanese isolates contain a G at position −99 and a T at position −2, instead of the A and C, respectively, common to all of the other sequences in Fig. 1. The HCJ-4, but not the HCJ-1, Japanese sequence reported by Okamoto et al. (28) contains the same G alteration at −99, but both contain the HCV-1 C at −2.

RT-PCR was initially applied to the detection of HCV by Weiner et al. (40–42) and has been used more recently by others (11, 12, 18, 19). However, primer sets used for PCR analysis have been inadvertently designed for segments of the HCV genome that are now known to vary significantly on the nucleotide level. This variation is particularly apparent when the nucleotide sequences of viral isolates from different parts of the world are compared. It is likely, therefore, that investigators have been unable to detect HCV in truly viremic samples because of the viral sequence variation. A recent report (27) has also suggested that the 5PUT region may be used for RT-PCR detection of HCV in Japanese samples.

For HCV immunoassay-positive blood donors, the level of RT-PCR positivity with 36-37b primers is lower than that observed by Weiner et al. (40, 42). Also, the difference between the responses observed for the 5PUTs/a and 36-37b primer sets is even greater than anticipated on the basis of sequence variation. We cannot discount the possibility that our cDNA synthesis PCR and/or Southern detection protocols for the NS3 region are not as well optimized as for the

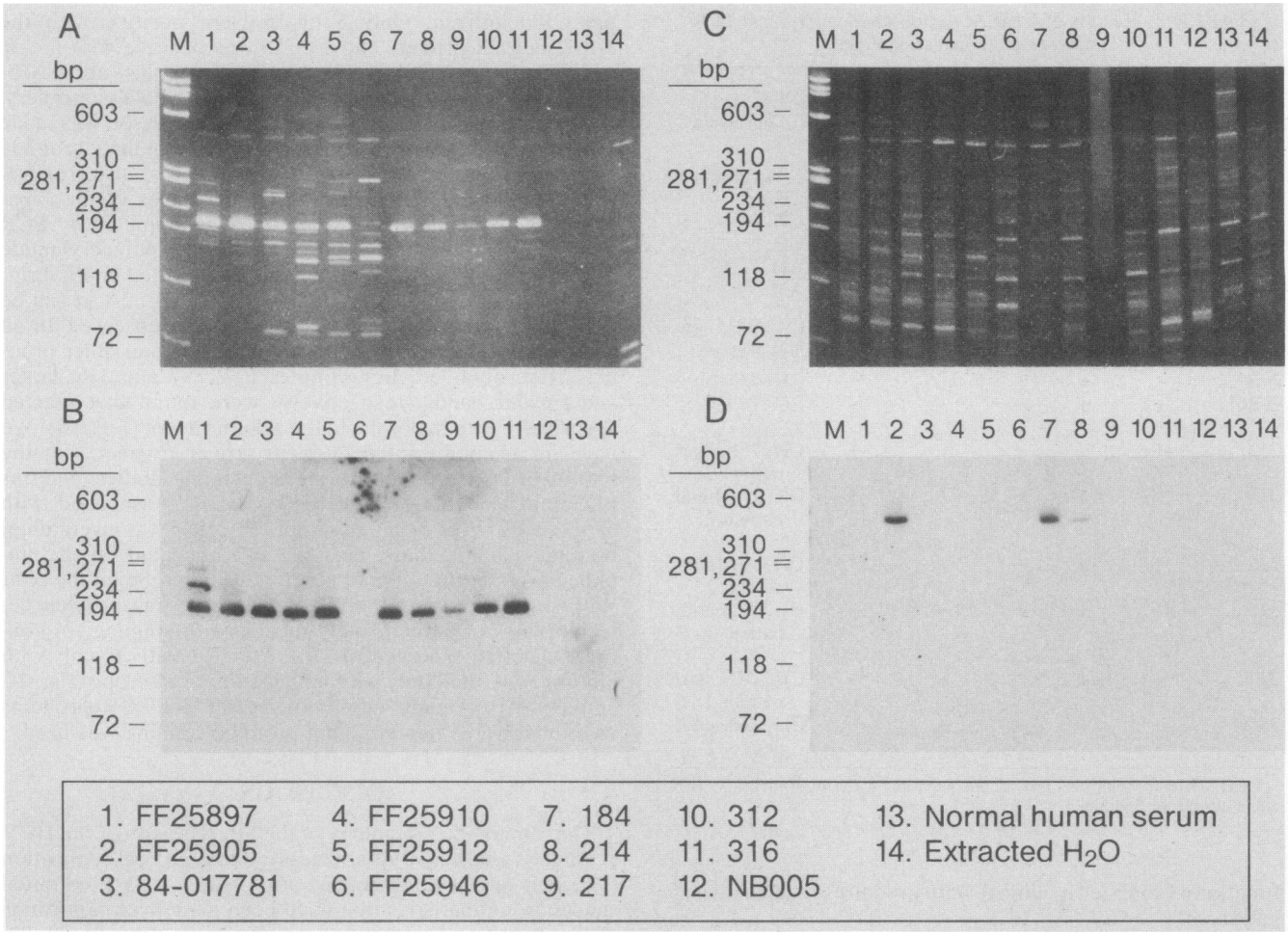

FIG. 2. Ethidium bromide-stained gels and Southern blots of RT-PCR reactions for U.S. and Japanese HCV RNA in human plasma samples. The samples used for all of the panels were identical and are listed in the enclosed box. Samples 1 to 6 were from the United States, while samples 7 to 12 were of Japanese origin. The 5PUT s/a and 36-37b primers were employed for the analyses presented in panels A and B and C and D, respectively. Panels A and C are photographs of ethidium bromide-stained gels, while panels B and D are autoradiographs of blots from the same gels. Panel B was probed with the radioactively labelled oligonucleotide probe indicated in Table 1. Panel D was probed with a nick-translated probe from a plasmid which contained HCV sequences inside PCR primers 36 and 37b.

5PUT region. It is also conceivable that the 5PUT portion of the genome is present at elevated levels or is inherently more stable (13).

For the 5PUTs/a RT-PCR-negative samples, it is not possible to discern whether (i) the virus is present at a level below the assay detection limit, (ii) the virus is sequestered in the liver or elsewhere, or (iii) the infection has been fully resolved. Loss of the PCR signal after interferon treatment could indicate viral clearance (18); however, we have recently identified an individual who became PCR positive again 3 months after apparent loss of signal upon interferon therapy (unpublished data). We are looking further into this phenomenon.

Given the extraordinary conservation in the 5PUT region of HCV, we suggest that the low 5PUTs/a RT-PCR positivity rate for immunologically positive blood donors is due to poor sensitivity, the life cycle of the virus, or RNA degradation during storage. In contrast to PCR for DNA targets, in which 1- to 10-molecule sensitivity has been reported (10, 14, 29), RT-PCR is typically sensitive to 2×10^2 to 1×10^4 RNA target molecules (34, 38). Estimates of the number of HCV genomes per milliliter of human plasma have been made from infectious titers in chimpanzees (3) and by dilutional PCR titration of HCV cDNA generated from human samples (34). The results of both types of analysis suggest a titer ranging from 10^2 to over 10^6 virions per ml. As a result, it is possible that some samples contain RNA at a level below the detection limit of even RT-PCR.

Although PCR detection of the HCV genome in clinical samples is a powerful and sensitive technique, it is difficult to perform reliably (24), highly user dependent, and very time consuming. In our studies, one individual could perform 20 to 40 PCR analyses in 4 to 7 days. Also, although it is possible to obtain semiquantitative information from PCR analysis by using either internal standards or dilutional titration, these methods are very labor intensive and require multiple analyses for each patient (34, 38). As has been demonstrated for the monitoring of interferon therapy for hepatitis B virus infection (7, 29), quantitative HCV nucleic acid determination is anticipated to aid in determining prognosis and efficacy of therapy. By using the 5PUT sequence of HCV, we are developing a quantitative HCV RNA

detection system (analogous to the 2-h hepatitis B virus assay reported elsewhere [35, 36]) based on a signal amplification method that employs branched synthetic DNA (15) and chemiluminescence.

We have compared the 5PUT nucleotide sequences of HCV isolates from around the world. This HCV signature sequence has been used to design PCR primers that permit us to detect the virus in human plasma samples from the United States and Japan with better specificity and sensitivity than previously possible. The qualitative analysis may be particularly useful for determination of HCV infection in acute infections prior to seroconversion. In addition, the use of primers or probes from the 5PUT sequence should facilitate studies requiring direct HCV detection, such as monitoring of therapeutic regimens.

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